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Effects of oncogenic mutations in *Smoothed* and *Patched* can be reversed by cyclopamine

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Basal cell carcinoma, medulloblastoma, rhabdomyosarcoma and other human tumours are associated with mutations that activate the proto-oncogene *Smoothed* (*SMO*) or that inactivate the tumour suppressor *Patched* (*PTCH*). *Smoothed* and *Patched* mediate the cellular response to the Hedgehog (Hh) secreted protein signal, and oncogenic mutations affecting these proteins cause excess activity of the Hh response pathway^{1,2}. Here we show that the plant-derived teratogen cyclopamine, which inhibits the Hh response^{3,4}, is a potential 'mechanism-based' therapeutic agent for treatment of these tumours. We show that cyclopamine or synthetic derivatives with improved potency block activation of the Hh response pathway and abnormal cell growth associated with both types of oncogenic mutation. Our results also indicate that cyclopamine may act by influencing the balance between active and inactive forms of *Smoothed*.

Whereas embryonic loss of Sonic hedgehog (Shh) signalling can result in cyclopia and other developmental defects⁵, inappropriate activation of the Shh response pathway is associated with several types of human tumour^{6–13}. Current approaches to treatment of such neoplastic disorders are limited by the cytotoxic effects of therapeutic agents on proliferating tissues. Alternative 'mechanism-based' approaches specifically targeting abnormally active signalling pathways in defined types of cancer¹⁴ might avoid such toxicity,

particularly if the pathways in question functioned primarily in embryonic development and were not required for survival in adults. Cyclopamine, a plant steroidal alkaloid, induces cyclopia in vertebrate embryos¹⁵ and has been shown to act by inhibiting the cellular response to the Shh signal^{3,4}. To evaluate the therapeutic potential of cyclopamine for the treatment of Hh-pathway-associated disorders we investigated the mechanism by which cyclopamine acts.

Cellular responses to the Hh signal are controlled by two transmembrane proteins, Smo and Ptch, which are predicted to have seven and twelve transmembrane spans, respectively. Genetic and biochemical evidence indicates that Ptch suppresses the activity of Smo, and that binding of Hh to Ptch relieves this suppression^{1,2}, allowing activation of downstream targets through the Ci/Gli family of transcriptional effectors^{16–19}. As our previously described Hh signalling assay used *Drosophila melanogaster* cultured cells and was not sensitive to cyclopamine (ref. 18 and data not shown), we screened several vertebrate cell lines for a sensitive transcriptional response to palmitoyl- and cholesteryl-modified ShhN polypeptide (ShhN_p)²⁰ (Fig. 1a) using a Gli-dependent luciferase reporter²¹. Among several responsive fibroblast cell lines, NIH-3T3 mouse embryonic fibroblasts, which respond with a 20–150-fold induction of luciferase activity (Fig. 1b), were selected for all further studies except those requiring particular genetic backgrounds. Treatment of the cells with cyclopamine completely abolished the response to ShhN_p (Fig. 1c). To confirm the validity of this assay, we analysed the

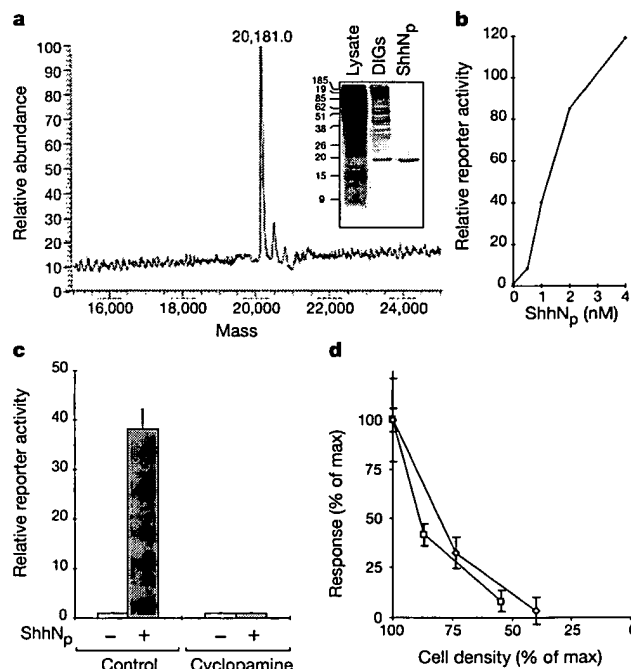


Figure 1 NIH-3T3 fibroblasts respond to ShhN_p and are sensitive to cyclopamine. **a**, Sonic hedgehog signalling domain was purified by isolating detergent-insoluble glycolipid complexes (DIGs) from 293 cells expressing full-length Shh³, followed by immunoaffinity chromatography. The purified species corresponds to palmitoyl- and cholesteryl-modified ShhN polypeptide (ShhN_p) from mass spectrometry analysis. Inset, SDS-polyacrylamide gel electrophoresis analysis of lysate, DIGs and purified ShhN_p. The responsiveness of NIH-3T3 cells to Shh (**b**) and cyclopamine (**c**) was analysed by transfection with Gli-luc reporter and Renilla luciferase control followed by treatment with ShhN_p (4 nM or as indicated) and/or cyclopamine (5 μM) for 2 d. Luciferase activities normalized relative to control. For time response, see Supplementary Information. **d**, The effect of cell density on Shh pathway activity was analysed by plating cultures of stable Shh reporter lines Shh-LIGHT (squares) or SmoA1-LIGHT (diamonds) cells in quadruplicate to 96-well plates in a series of twofold dilutions. The Shh-LIGHT cells were treated with 4 nM ShhN_p, and Gli-luc reporter activity (relative to equally dense Shh-LIGHT control culture) and relative cell density (% of maximum Renilla activity) were assayed after 24 h. Error bars, 1 s.d.

effects of overexpression of known pathway components, treatment with known pathway inhibitors, or both (Table 1). The main findings of *Drosophila* and mouse genetic analyses were confirmed, indicating that NIH-3T3 cells provide a faithful and physiologically meaningful model for analysis of the Shh signalling pathway. Interestingly, for a full response to ShhNp, cells had to be assayed after reaching saturation density (Fig. 1d).

The steroidal nature of cyclopamine and its ability to disrupt cholesterol synthesis or transport^{3,22,23} indicated that it might affect the action of Ptch, which contains an apparent sterol-sensing domain^{1,22}. Having established the general characteristics of Shh response and cyclopamine inhibition in mouse embryonic fibroblasts, we assayed fibroblasts derived from *Ptch*^{-/-} mouse embryos for cyclopamine sensitivity. Mice lacking functional Ptch show widespread transcriptional activation of targets of Shh signalling, including *Ptch* itself²⁴. As β -galactosidase is expressed under the control of the *Ptch* promoter in these cells, its expression can be used to assay the state of Shh pathway activity. Addition of cyclopamine to *Ptch*^{-/-} cells significantly suppressed β -galactosidase expression (Fig. 2a) and the activity of the Gli-luc reporter (see Supplementary Information), indicating that cyclopamine can inhibit Shh pathway activity in the absence of Ptch function. In contrast, cyclopamine failed to prevent pathway activation induced by Gli2 overexpression (Table 1).

These results suggest that Hh-pathway-related tumours associated with loss of Ptch function might respond to treatment with cyclopamine, and that the target of cyclopamine action is likely to be a pathway component that functions between Ptch and the Gli proteins. It is unlikely that Ptch2 is the target of cyclopamine action in *Ptch*^{-/-} cells, as Ptch is the main regulator of Shh pathway activation in embryos²⁴ and Ptch2 activity appears not to be expressed in *Ptch*^{-/-} cells (data not shown).

To investigate further the mechanism of cyclopamine action, we transfected NIH-3T3 cells transiently with both luciferase reporter and Smo complementary DNA, and found that overexpression of Smo in the absence of Shh induces reporter expression about tenfold. This Shh-independent activation of the response pathway can be suppressed by 5 μ M cyclopamine (Fig. 2b), consistent with a target of cyclopamine action downstream of Ptch and with a mechanism that does not involve direct interference with Shh binding. Cyclopamine at this concentration had little effect on

reporter expression induced by the oncogenic Smo mutants W539L¹³ (SmoA1; Fig. 2b) and S537N¹¹ (SmoA2; data not shown); cells from *Ptch* mutant embryos gave similar results (see Supplementary Information).

These results indicate that cyclopamine may act upon Smo, and that activating mutations render Smo proteins resistant. An alternative interpretation would be that activated Smo proteins produce a high abundance of a downstream component and that a high cyclopamine level is required to suppress the increased concentration of this component. This alternative model, however, would predict that intermediate or low levels of pathway activation by oncogenic Smo proteins expressed at low levels should be subject to cyclopamine inhibition; on the contrary, we observe that cyclopamine resistance is sustained under these conditions (Fig. 2b). In addition, cyclopamine resistance is not observed in cells expressing high levels of wild-type Smo activated by maximal Shh stimulation (not shown), again suggesting that cyclopamine does not act upon a component downstream of Smo.

The oncogenic SmoA1 protein has been reported to resist suppression by Ptch²⁵, indicating that oncogenic Smo proteins may not be subject to normal regulation. We found, however, that this resistance is partial. The activating effects of SmoA1 or SmoA2 can be completely inhibited by transfection of a 9-to-1 ratio of a Ptch construct or of *Ptch*-CTD, which encodes a carboxy-terminally deleted protein expressed at a higher level²⁶ (Fig. 2c and data not shown). We also found that SmoA1, thus inhibited by Ptch-CTD, responds well to stimulation by ShhNp. Under these circumstances,

Table 1 Luciferase activity from a Gli-dependent reporter as induced by combinations of Shh pathway inducing and suppressing treatments

Inducer	Suppressor					
	None	Ptc	Cyclopamine	Forskolin	PKA ^a	Gli3-N
None	-	-	-	-	-	-
ShhNp	+++	+	-	-	-	-
Smo	+	-	-	-	-	ND
Activated Smo	+++	+	+	-	-	-
Gli2	++	++	++	++	+	-

The indicated constructs were transfected alone or in combination (1:1 ratio) to NIH-3T3 cells, followed by treatment with ShhNp (4 nM), cyclopamine (5 μ M) or forskolin (100 μ M) for 2 days. Gli3-N, Gli3 truncated at residue 700, generating a repressor form. PKA^a, constitutively active protein kinase A catalytic subunit.

^aHigher dose can completely suppress (see Figs 2, 3).

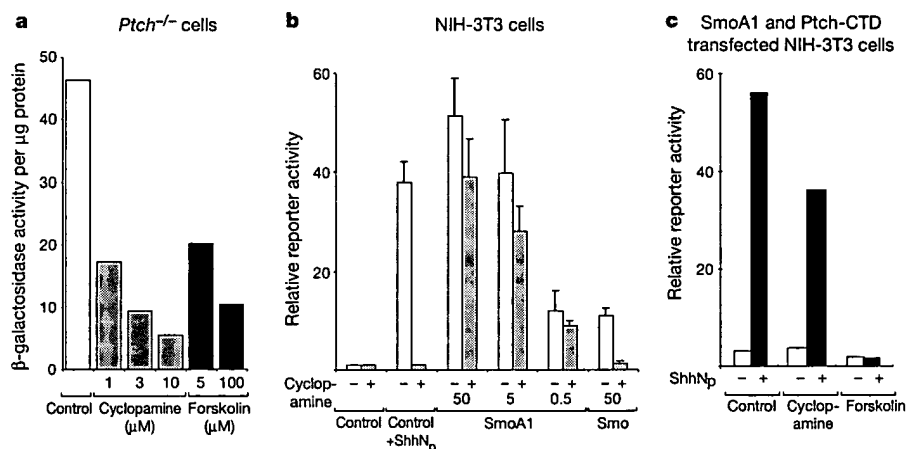


Figure 2 Cyclopamine acts downstream of Ptch by inhibiting Smo activity. **a**, Cyclopamine and forskolin can inhibit Hh pathway activity in *Ptch*^{-/-} cells (measured as expression of β -gal activity from the *Ptch* locus after 3 d of treatment). **b**, Sensitivity of wild-type and oncogenic Smo to cyclopamine was determined by transfecting NIH-3T3 cells with Gli-luc reporter and the w/w ratio of the expression vector indicated, followed by treatment with 5 μ M cyclopamine for 2 d. Error bars, 1 s.d. The leftmost four bars are as in Fig. 1c.

c, Activation of pathway by oncogenic Smo is inhibited by high amounts of Ptch (compare with **b**); ShhNp restores pathway activity even in the presence of cyclopamine. NIH-3T3 cells were transfected with reporter, Ptch-CTD and SmoA1 (Ptch:Smo DNA ratio 9), and treated with ShhNp (2 nM), cyclopamine (5 μ M) and/or forskolin (100 μ M) as indicated for 2 d. Data from a representative experiment are shown.

induction of the Gli-responsive reporter is resistant to 5 μ M cyclopamine (Fig. 2c), which would normally abolish Shh signaling. These results indicate that activated Smo molecules in the presence of sufficient Ptc can contribute to an essentially normal, albeit cyclopamine-resistant, response to the Shh signal.

The finding that oncogenic Smo is regulated by high levels of Ptc indicates that it might also be subject to regulation by high levels of cyclopamine. To circumvent the cytotoxic effects of cyclopamine concentrations greater than 10 μ M, we tested several chemically synthesized cyclopamine derivatives. As seen in Fig. 3a, the cyclopamine derivative 3-keto, *N*-aminoethyl aminocaproyl dihydrocin-

namoyl cyclopamine (KAAD-cyclopamine; see Methods), had 10–20-fold higher potency than cyclopamine in inhibition of β -galactosidase expression in p2^{Ptc^{-/-}} cells, with similar or lower toxicity (Fig. 3a). This compound also had greater potency in suppression of ShhN_p-induced pathway activity (Fig. 3b). Importantly, it completely suppressed SmoA1-induced reporter activity at a concentration around tenfold higher than that required for suppression of pathway activation induced by ShhN_p (Fig. 3b).

As activation of the Hh response pathway is associated with

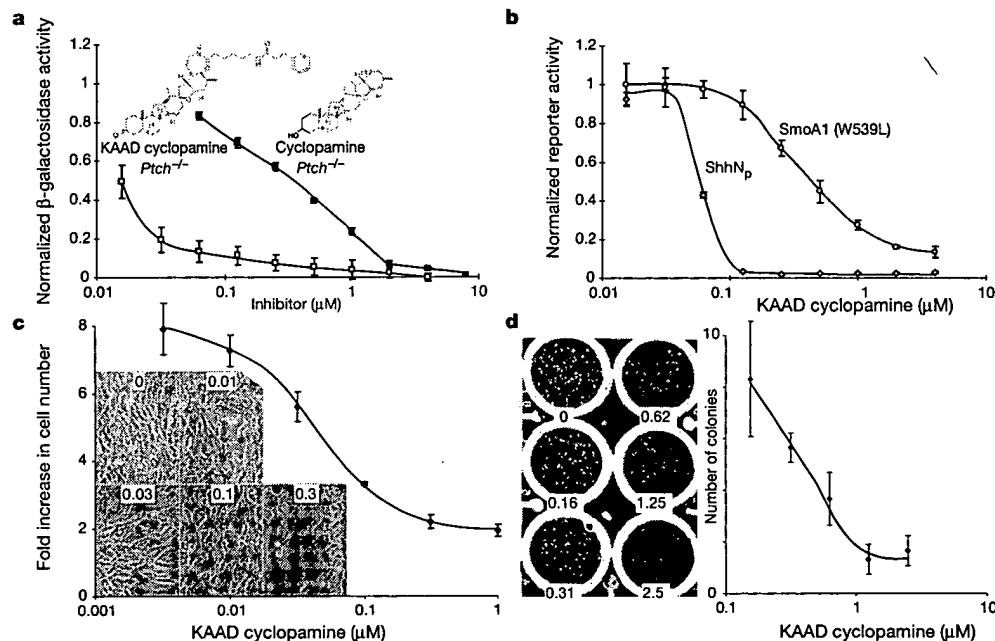


Figure 3 Reversal of pathway activation and abnormal cell growth caused by oncogenic mutations in Ptc and Smo. **a**, Confluent cultures of p2^{Ptc^{-/-}} cells were treated with cyclopamine (filled squares) or KAAD-cyclopamine (open squares) for 2 d. β -galactosidase activity was determined relative to cell mass (treated duplicate plate; Cell Titer 96AQ; Promega). Significant toxicity was not observed. **b**, Shh-LIGHT2 (diamonds) and SmoA1-LIGHT (circles) cells were treated with 4 nM ShhN_p (Shh-LIGHT2) and KAAD cyclopamine (both lines) for 2 d. **c**, p2^{Ptc^{-/-}} cells were cultured in 2% calf serum in the

presence of KAAD cyclopamine for 7 d (triplicate wells) and cell number was determined with a Coulter counter. Inset, micrographs of treated cells. **d**, SmoA1-LIGHT cells were plated in soft agar with the indicated concentrations of KAAD-cyclopamine. Left, ethidium-bromide-stained colonies after 17 d. Right, number of colonies (>150 μ m) per field after 10 d. Error bars, 1 s.d. Relative reporter activities in **a**, **b** are normalized to maximum.

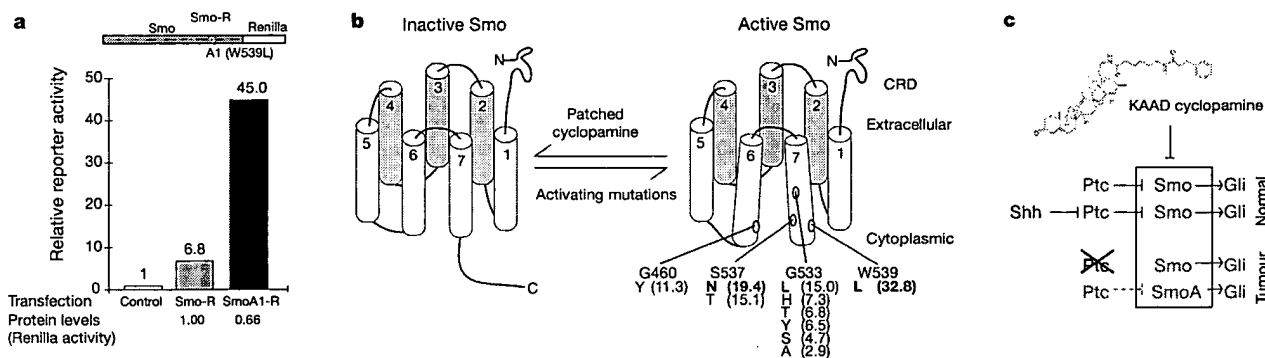


Figure 4 Mechanism of cyclopamine reversal of oncogenic Hh pathway activation. **a**, NIH-3T3 cells were transfected with Gli-luc reporter and a construct encoding the indicated Smo–Renilla luciferase fusion protein. Shh pathway activity and Smo protein levels were measured as firefly and Renilla luciferase activities relative to β -galactosidase transfection control, respectively. Expression levels of epitope-tagged Smo and SmoA1 also were similar (data not shown). **b**, A two-state model of Smo function and cyclopamine action. The states may reflect conformational change (as shown), distinct subcellular localizations or states of covalent modification. Below, summary of oncogenic^{11,13} (bold) and other activating mutations in Smo. The potency of each mutant protein in pathway activation relative to wild-type Smo (value set at 1; ShhN cotransfection, 21.1) is in

parentheses. Relative potencies were determined at 10% w/w effector plasmid. **c**, Cyclopamine inhibition of the Hh response pathway. Under physiological conditions the inactive state of Smo (red) is maintained by active Ptc (green), leading to lack of Gli-mediated transcription (red). Shh ligand activates the pathway by binding to and converting Ptc to its inactive state (red), which in turn permits activity of Smo (green) and results in Gli-mediated transcription (green). In tumours, Gli-mediated transcription is ligand-independent owing to loss of Ptc function (Ptc) or constitutive activation of Smo (SmoA). Cyclopamine inhibits the Hh response pathway and abnormal growth by inhibiting Smo activity.

neoplastic transformation in various types of tumour, we investigated the growth properties of response-activated cells in low serum or soft agar²⁷, conditions generally considered to reveal neoplastic transformation. As seen in Fig. 3c, p2^{Ptch-/-} cell growth in low serum was markedly inhibited by addition of KAAD-cyclopamine, with 50% of maximal inhibition at ~50 nM. We also found that SmoA1-LIGHT cells, a cell line expressing SmoA1 clonally derived from NIH-3T3 cells, can form colonies in soft agar medium. Addition of KAAD-cyclopamine markedly inhibited colony growth, although ~500 nM was required for 50% inhibition (Fig. 3d). This concentration was higher than that required for 50% maximal inhibition of p2^{Ptch-/-} cell growth, consistent with the higher amount of KAAD-cyclopamine required to block activation of the Hh response pathway by oncogenic Smo.

The activity of other signalling pathways headed by seven-transmembrane-domain receptors is determined by the balance between active and inactive forms of the receptor^{28,29}. Consistent with such a model for Smo, the level of Renilla-luciferase- or epitope-tagged Smo in transfected cells is not significantly affected by the presence of an oncogenic mutation, despite greatly elevated reporter activity (Fig. 4a). These results indicate that oncogenic Smo may have a higher intrinsic ability to activate the pathway, and that wild-type Smo may exist in a balance between active and inactive forms^{28,29}. Whereas cyclopamine and Ptch activities would shift this balance towards the inactive state, oncogenic mutations might exert the opposite effect, and this could account for the higher levels of Ptch and cyclopamine activity required to suppress oncogenic Smo. To test this model, we identified eight additional mutations in an extensive mutagenesis screen for activated Smo proteins (Fig. 4b). All the activating Smo mutants were cyclopamine resistant, and the level of resistance positively correlated with increased potency (see Supplementary Information), consistent with a model in which cyclopamine affects the balance between activity states of Smo (Fig. 4b).

The transition of G-protein-coupled receptors with seven transmembrane (TM) domains from the inactive to the active state is thought to involve a conformational shift in which the cytoplasmic ends of the TM6 and TM7 helices tilt outwards, exposing a binding pocket for a downstream signalling molecule, the G α subunit²⁹. Our mutational analysis of Smo is consistent with such a conformational shift, as eight of the Smo-activating mutations introduce bulkier side chains at G533 and S537. Assuming an α -helical conformation for TM7, these substitutions all protrude from the same face of the helix. Furthermore, as multiple distinct substitutions at each of these two residues result in activation (Fig. 4b), Smo would appear to be activated by alteration of helix-packing interactions rather than by creation or disruption of a single critical interaction. A conformational shift of the TM7 helix with respect to other TM helices is suggestive of the type of conformational transition postulated for activation of G-protein-coupled receptors and raises the possibility that conformation-based transduction has been conserved in the evolution of seven-transmembrane-domain receptors.

Activation of the Hh response pathway has been linked to several types of human tumour. For example, patients with basal cell nevus syndrome (also termed Gorlin syndrome), an autosomal dominant disorder associated with heterozygous loss-of-function mutations in *PTCH*, display increased incidence of many tumours, most notably basal cell carcinoma (BCC), medulloblastoma, rhabdomyosarcoma and fibrosarcoma⁶⁻⁸. In addition, loss-of-function mutations in *PTCH* or activating mutations in *SMO* are found in around 40% of sporadic BCC and 25% of primitive neuroectodermal tumours^{6,9-13}. Our results indicate that cyclopamine inhibits the Shh pathway by antagonizing Smo and that the activation of the Hh response pathway by either type of oncogenic mutation is blocked by cyclopamine or its derivatives (Fig. 4c). Levels of cyclopamine or the related compound jervine required to phenocopy the embryonic malformations in *Shh*^{-/-} embryos are tolerated by pregnant females

of various species, indicating that it might be possible to use these compounds or their derivatives without severe toxicity in non-pregnant adults to reverse activation of the Shh response pathway for therapeutic purposes. □

Methods

Synthesis of KAAD-cyclopamine and purification of ShhN_p

KAAD-cyclopamine was synthesized from cyclopamine by a route to be described elsewhere (J.K.C. and P.A.B., manuscript in preparation). Cholesterol- and palmitate-modified mouse Sonic hedgehog signalling domain ShhN_p was purified as described in Supplementary Information (see also Fig. 1a).

cDNA cloning, mutagenesis and constructs

A mouse full-length *Smo* cDNA was isolated using standard methods on the basis of homology to rat and human *Smo* sequences. Of two described activating mutations in human *Smo*^{13,25}, one (SmoM2; corresponds to W539L in mSmo and referred to here as SmoA1) activated mouse Smo and the other (SmoM1; corresponding to R565Q) did not. For mutagenesis screens, Ala substitutions were introduced at more than 20 conserved or hydrophilic residues within Smo transmembrane segments; in addition, selected residues were changed to 16 different amino acids (all except W, M, K and E) using a degenerate oligonucleotide and screened by transfection of a pool of clones. We secondarily screened 53 (G533) or 37 (G460) individual clones to identify mutations at these positions that activate Smo. Expression constructs used: pRK5 for full-length mouse Ptch, C-terminally truncated Ptch-CTD, Gli3(1-700) repressor¹⁹ and constitutively active PKA¹⁸ cDNAs, and pGE (transient transfections) or pcDNA3.1+hygro (stable lines; Invitrogen) for the various *Smo* cDNAs. pGE was derived from pEGFP-C1 (Clontech) by removal of the enhanced green fluorescent protein cDNA. Renilla luciferase was fused to the C terminus of Smo. These fusion protein constructs had similar activity to corresponding untagged constructs in the NIH-3T3 assay.

Cell lines

Primary fibroblasts lacking functional Ptch (*Ptch*^{-/-}) and a clonal subline (p2^{Ptch-/-}) were derived from *Ptch*^{-/-} mouse embryos. The NIH-3T3 cell clones Shh-LIGHT and Shh-LIGHT2 stably incorporating the Gli-luc reporter and TK-Renilla vectors were established by co-transfection with vector encoding G418 resistance (pSV-Neo). SmoA1-LIGHT cells are a clonal subline of Shh-LIGHT expressing oncogenic Smo (verified by immunoblotting).

Transfection and reporter assays

Confluent cultures of NIH-3T3 cells were plated at 1:6 dilution to 24- or 96-well plates. On the next day, we transfected the cells with Renilla luciferase (pRL-TK or pRL-SV40; Clontech) or β -galactosidase transfection control (10% w/w DNA), Gli-luc reporter (40%) and the constructs indicated (50%) using Eugene 6 (Roche) transfection reagent (250 ng (24-well plate) or 100 ng (96-well plate) DNA per well, 3:1 ratio (v/w) of reagent to DNA). After the cells had reached saturation density (1-2 days), they were changed to low-serum medium (0.5% bovine calf serum) and treated with the reagents indicated for 1-2 days. All reporter assays were normalized for transfection efficiency using Renilla luciferase or β -galactosidase transfection control (luminometric detection, Promega dual luc; Tropix galacto-light).

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Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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The APC tumour suppressor has a nuclear export function

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The adenomatous polypoid coli (APC) protein is mutated in most colorectal tumours¹. Nearly all APC mutations are truncations, and many of these terminate in the mutation cluster region located halfway through the protein^{2–4}. In cancer cells expressing mutant APC, β -catenin is stabilized^{5,6} and translocates into the nucleus to act as a transcriptional co-activator of T-cell factor⁷. During normal development, APC also promotes the destabilization of β -catenin and *Drosophila* Armadillo^{8–11}. It does so by binding to the Axin complex which earmarks β -catenin/Armadillo for degradation by the proteasome pathway¹². APC has a regulatory role in this process^{13,14}, which is poorly understood. Here we show that APC contains highly conserved nuclear export signals 3' adjacent to the mutation cluster region that enable it to exit from the nucleus. This ability is lost in APC mutant cancer cells, and we provide evidence that β -catenin accumulates in the nucleus as a result. Thus, the ability of APC to exit from the nucleus appears to be critical for its tumour suppressor function.

Adenomatous polypoid coli proteins are found in several subcellular compartments of mammalian and *Drosophila* cells including the cytoplasm, nucleus and adhesive cadherin/catenin junctions^{9,10,15}. To identify the targeting domains for these compartments, we tagged various fragments of the ubiquitously expressed *Drosophila* APC, called E-APC/dAPC2, with green fluorescent protein (GFP) and expressed these in transgenic fly embryos and in monkey COS cells. The subcellular distribution of GFP-E-APC is indistinguishable from that of endogenous E-APC in embryos⁹ (data not shown). In COS cells transfected with GFP-E-APC, we see green fluorescence in the cytoplasm, concentrated at the plasma membrane (to be described elsewhere), but also some in the nucleus (Fig. 1a). Unexpectedly, an amino-terminal fragment of E-APC (ARDcore; Fig. 2a) accumulates in the nucleus (Fig. 1b). Evidently, E-APC is capable of entering the nucleus by means of its N terminus. We have not studied this further, but we note that this N terminus spans the highly conserved Armadillo repeat domain (ARD). β -catenin contains a closely related ARD that mediates its nuclear import independently of the Ran/Importin machinery^{16,17}.

In contrast, carboxy-terminal fragments of E-APC (Cterm1 and 2; Fig. 2a) are efficiently excluded from the nucleus (Fig. 1c), more so than the full-length protein. Thus, the C terminus of E-APC either contains a cytoplasmic anchoring domain or an efficient nuclear export signal (NES). To distinguish between these possibilities, we tested our GFP constructs by treating transfected cells with leptomycin B (LMB), a highly specific drug that inhibits nuclear export by directly blocking the nuclear export receptor CRM1 (refs 18–20). Indeed, this results in even distribution of Cterm1 and Cterm2 throughout cytoplasm and nucleus (Fig. 1d).

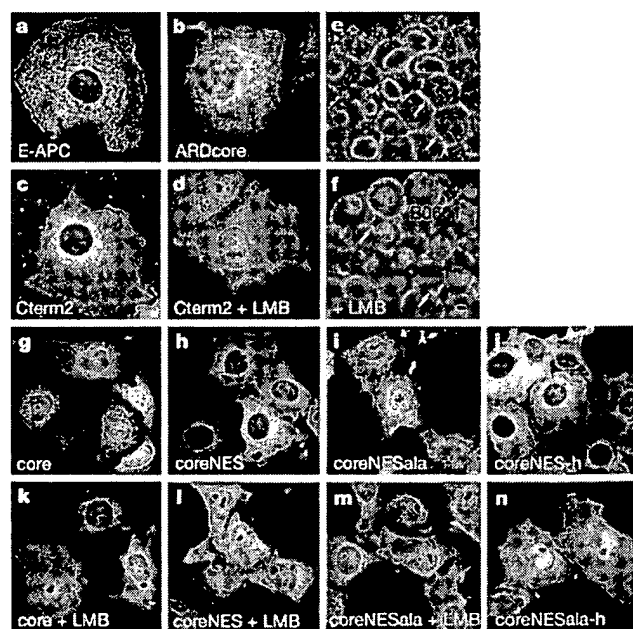


Figure 1 Nuclear export sequences within *Drosophila* and human APC. **a–d**, COS cells transfected with GFP-tagged constructs as indicated, without (**a–c**) or with (**d**) LMB. Cterm1 was excluded from nuclei at least as efficiently as Cterm2 (**c**); this exclusion, like that of Cterm2 (**d**), was blocked by LMB. **e, f**, Face-on views of 4–5-hour-old *Drosophila* embryos, stained with anti-E-APC (green) outlining the apical cellular junctions (arrows), and anti-lamin (red) outlining the nuclear envelopes. Note the nuclear accumulation of E-APC after LMB treatment (**f**). **g–n**, COS cells transfected with coreNES constructs. **g, k**, ARDcore; **h, l**, coreNES from E-APC 20R4; **i, m**, coreNESala from E-APC 20R4; **j, n**, coreNES from human APC 20R3; **n**, coreNESala from human APC 20R3. All coreNES constructs described in the text behaved essentially the same. Cells in **k–m** were treated with LMB; the staining patterns of the human constructs (**j, n**) after LMB treatment are similar to those in **k–m**.